Characterization of Neutralizing Antibody Responses Elicited by Clade A Envelope Immunogens Derived from Early Transmitted Viruses[▽]

Zane Kraft,¹ Katharine Strouss,¹ William F. Sutton,^{1†} Brad Cleveland,² For Yue Tso,^{2‡} Patricia Polacino,³ Julie Overbaugh,⁴ Shiu-Lok Hu,^{2,3} and Leonidas Stamatatos^{1,5*}

Seattle Biomedical Research Institute, Seattle, Washington 98109¹; Department of Pharmaceutics,² Washington National Primate Research Center,³ Seattle, Washington 98195; Division of Human Biology, Fred Hutchinson Cancer Research Institute, Seattle, Washington 98109⁴; and Department of Pathobiology, University of Washington, Seattle, Washington 98109⁵

Received 22 February 2008/Accepted 1 April 2008

The vast majority of studies with candidate immunogens based on the human immunodeficiency virus envelope (Env) have been conducted with Env proteins derived from clade B viruses isolated during chronic infection. Whether non-clade B Env protein immunogens will elicit antibodies with epitope specificities that are similar to those of antibodies elicited by clade B Envs and whether the antibodies elicited by Envs derived from early transmitted viruses will be similar to those elicited by Envs derived from viruses isolated during chronic infection are currently unknown. Here we performed immunizations with four clade A Envs, cloned directly from the peripheral blood of infected individuals during acute infection, which differed in lengths and extents of glycosylation. The antibody responses elicited by these four Envs were compared to each other and to those elicited by a well-characterized clade B Env immunogen derived from the SF162 virus, which was isolated during chronic infection. Only one clade A Env, the one with the fewer glycosylation sites, elicited homologous neutralizing antibodies (NAbs); these did not target the V1, V2, or V3 regions. In contrast, all four clade A Envs elicited anti-V3 NAbs against “easy-to-neutralize” clade B and clade A isolates, irrespective of the variable region length and extent of glycosylation of the Env used as an immunogen. These anti-V3 NAbs did not access their epitopes on homologous and heterologous clade A, or B, neutralization-resistant viruses. The length and extent of glycosylation of the variable regions on the clade A Env immunogens tested did not affect the breadth of the elicited NAbs. Our data also indicate that the development of cross-reactive NAbs against clade A viruses faces similar hurdles to the development of cross-reactive anti-clade B NAbs.

Efforts to develop a protective vaccine against human immunodeficiency virus (HIV) are hindered by the limited potential of currently available HIV Env-based immunogens to elicit broadly cross-reactive neutralizing antibody (NAb) responses. Initial immunization studies were conducted with soluble monomeric gp120 proteins, which elicit primarily homologous NAb responses (10, 28, 29, 42). Subsequent HIV Env immunogen design efforts focused on the engineering of stable soluble trimeric gp140 constructs (5, 13, 22, 47, 52, 54, 61, 66, 67). Such constructs elicit somewhat broader anti-HIV NAb responses than the gp120 immunogens, but the breadth of these responses is still very narrow (2–4, 11, 17, 19–21, 23, 27, 38, 50, 64, 65, 68, 71).

The above mentioned studies were conducted with Envs derived from clade B viruses isolated during the chronic phase of infection (“late” viruses), such as HxB2, ADA, YU2, JRFL, and SF162. Whether Envs derived from “early” and “late” viruses differ, or not, in their immunogenic properties is not yet known. The Envs of viruses present early in infection have been shown to have shorter V1-V2 loops with less glycosylation

than chronic-stage variants (16, 18). Although smaller V1-V2 Env regions and fewer glycosylation sites are in general related to a greater susceptibility of HIV to neutralization (1, 8, 12, 16, 18, 31, 32, 34, 40, 41, 43, 45, 46, 48, 49, 60, 69), it has not yet been determined whether Envs with smaller V1-V2 regions and fewer glycosylation sites will elicit different types of antibodies than those elicited by Envs with longer V1-V2 regions or Envs that are more extensively glycosylated.

Clade A infections predominate in central and eastern Africa and the countries of the former Soviet Union and account for an estimated 25% of global HIV-1 infections (9). Thus, clade A viruses are an important target for an effective global HIV vaccine. Very little is however known about the immunogenic properties of clade A Envs. Clade A Env immunogens derived from two viruses (92RW020 and 92UG037) isolated during chronic infection have been previously included in polyvalent vaccine formulations (55, 56, 62), but their individual immunogenic properties have not been examined. In the present study, we investigated the types of antibody responses elicited during immunization with four clade A Envs. These Envs were derived from viruses isolated from four acutely infected subjects (39). Variants were selected to represent Env sequences with different lengths and numbers of potential N-linked glycosylation sites in their variable regions, especially the V1-V2 region. Since, the length and glycosylation extent of the V1-V2 region have been linked to the overall neutralization phenotype of HIV, we examined whether these differences may affect the types of antibodies elicited during immuniza-

* Corresponding author. Mailing address: Seattle Biomedical Research Institute, 307 Westlake Avenue North, Seattle, WA 98109. Phone: (206) 256-7200. Fax: (206) 256-7229. E-mail: leo.stamatatos@sbri.org.

† Present address: Oregon Health & Science University, Beaverton, OR 97006.

‡ Present address: University of Nebraska, Lincoln, NE 68588.

[▽] Published ahead of print on 9 April 2008.

tion. In addition, these Envs were derived from viruses present a median of 35 days after infection (39) and thus provided the opportunity to examine the immunogenicity of Envs representing "early," recently transmitted variants.

The Env from the clade B SF162 virus was also included in the immunization study. This immunogen elicits high titers of homologous NAb responses in rabbits, guinea pigs, and macaques, but heterologous NABs of narrow breadth (2, 17). In this regard, the immunogenic properties of SF162 Env-derived immunogens are not different from those of Envs derived from other primary HIV-1 isolates such as JRFL or YU2 (3, 4, 20, 23, 27, 38), but with one potential exception (71). We therefore compared the NAb responses elicited by our four clade A Envs to each other and to those elicited by this clade B Env.

Overall, our study indicates that the antibodies elicited by these four clade A Env variants have a narrow breadth of cross-neutralizing activities that target the V3 loop, suggesting that the development of broadly reactive NABs against clade A viruses faces similar obstacles to those for clade B viruses.

MATERIALS AND METHODS

Envelope immunogens. The four clade A Envs used here were derived from four clade A clones (Q168, Q461, Q259, and Q769) obtained directly from the infected individuals during acute infection (39). The following Env clones were used to generate gp160 and gp140 immunogens: Q168a2, Q259d2.17, Q461e2, and Q769h5. With the exception of Q259d2.17, these clones are resistant to neutralization by heterologous clade A sera (6). The fifth Env used as an immunogen was derived from the clade B isolate, SF162 (57).

Recombinant vaccinia viruses. Recombinant vaccinia viruses were constructed as previously described (30) to express either the full-length (gp160) or the ectodomain (gp140) of the Env proteins of HIV-1 subtype B isolate SF162 and subtype A Q168a2, Q259d2.17, Q461e2, and Q769h5. Expression of the *env* gene is under the control of a synthetic early-late promoter (14). The transgenes were inserted into the thymidine kinase gene of vaccinia virus v-NY, which is a plaque-purified, replication-competent derivative of the New York City Board of Health strain of vaccinia virus (70). Expression of the transgenes was verified by Western blot analysis.

Purification of soluble gp140. The gp140 versions of these Envs were generated by introducing a stop codon immediately upstream of the transmembrane region of *env* and by eliminating by mutagenesis the primary and secondary gp120-gp41 cleavage sites, as previously described (58, 59). Recombinant gp140 proteins were purified as previously described for gp160 proteins (33), with the exception that infected cell culture supernatant, rather than vaccinia virus-infected cell lysate, was used as the starting material. Briefly, African green monkey kidney cells (BSC-40) were infected at a multiplicity of infection of 3 with recombinant vaccinia virus expressing the gp140 Env antigen of subtype A HIV-1 isolates described here. Cell culture supernatant was collected after 48 h of infection, cleared of cell debris by centrifugation, and passed through a lentil lectin affinity column. After removal of unbound material, gp140 was eluted from the lentil lectin column in the presence of 1 M methyl α -D-mannopyranoside. Eluate from the lentil lectin column was then passed through an S-200 Superdex size exclusion column. Peak fractions were collected, dialyzed against phosphate-buffered saline, and kept frozen until formulation with adjuvant for immunization. The purity of the final product is estimated to be 80 to 90% by scanning of Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, and the integrity of the gp140 protein is estimated to be >90% by Western blot analysis.

Peptides. The following peptides were used during epitope-mapping experiments. The SF162-derived peptides were TNLKNATNTKSSNWKEMDRGEIK (V1), NNNTRKSITIGPGRFYATGC (V3), and TTSIRNKMOKEYALF (V2Nt, derived from the amino-terminal [Nt] side of the V2 loop). The Q259d2.17-derived peptides were CTRPNNNTRKSVRIGPGQAF (V3Nt, derived from the amino-terminal side of the V3 loop), PGQAFYATDDIIGNIR QAYC (V3Ct, derived from the carboxy-terminal [Ct] side of the V3 loop), CYNVTKSDKITKDMQEEIKN (V1), EEIKNCSFNITTELDRKKQK (V2Nt, derived from the amino terminal side of the V2 loop), DKKQKVHSLFYRLD VVPMGG (V2crown, derived from the central region or "crown" of the V2

loop), and VPMGGKND SQYRLINCNTSA (V2Ct, derived from the carboxy-terminal side of the V2 loop).

Immunizations. Specific-pathogen-free New Zealand White rabbits (*Oryctolagus cuniculus*) of either sex, 5 to 7 months old, were immunized at 0 and 8 weeks by skin scarification with one of the five recombinant vaccinia viruses (5×10^7 PFU) expressing the gp160 form of the Envs described above. Animals were boosted at week 24 with 100 μ g of the corresponding Env gp140 produced in vaccinia virus-infected BSC-40 cells. The gp140 Envs were formulated in incomplete Freund's adjuvant and delivered intramuscularly. Sera were collected both prior to (pre-bleeds) and during immunization. The neutralizing activity of sera was evaluated 2 weeks following the recombinant gp140 immunization.

Neutralization assays. Single-round competent virions (pseudoviruses) were generated by cotransfecting 293T cells with Env-expressing DNA plasmid and a plasmid expressing the NL43 Luc⁺ Env⁻ backbone, as previously described (53). Pseudoviruses expressing the five vaccine Envs (SF162, Q168a2, Q259d2.17, Q461e2, and Q769h5) as well as heterologous clade B Envs derived from ADA, JRFL, SS1196, QH0692, 3988, BG1168, 6101, PAVO, and QH0515 (37) were generated.

Neutralizations were performed using TZM-bl cells as targets as previously described (17, 36, 53). Briefly, pseudovirus was first incubated for 1.5 h at 37°C with serially diluted heat-inactivated sera. The mixture was then added for 72 h to TZM-bl cells (3×10^3 cells per well of a 96-well plate). Triplicate wells per each serum dilution were used. The levels of cell-associated luciferase (measured as relative light units [RLU]) were determined in each well on a Fluoroskan Ascent fluorimeter, and the percentage of neutralization at every postimmunization serum dilution was determined against the same dilution of the corresponding preimmunization serum, using the following equation: $[(RLU_{\text{pre-bleed}} - RLU_{\text{immune}})/RLU_{\text{pre-bleed}}] \times 100$. The percentage of neutralization versus serum dilution was plotted, and the 50% inhibitory serum concentrations (IC_{50}) were determined.

Epitope mapping of vaccine-elicited serum antibody responses. Ninety-six-well enzyme-linked immunosorbent assay (ELISA) plates were coated with Env-derived peptides (1 μ g/ml) overnight at 37°C. The coated plates were blocked with Superblock (Pierce)–0.3% Tween. Serially diluted sera were added for 2 h at 37°C. Following washing, horseradish peroxidase-labeled protein G was added and incubated for 2 h at room temperature. The ELISA plates were washed, and 1-step Ultra TMB (tetramethyl benzidine; Pierce) was added to each well. Following 2 min of incubation at room temperature, the reaction was stopped with the addition of 0.5 N H₂SO₄. The optical density at 450 nm was determined.

Peptide competition during neutralization. Serial dilutions of postimmune sera (20 μ l) were incubated in duplicate with an equal volume of peptide (10 μ g/ml during this incubation step) for 1 h at 37°C. The mixture was then mixed with 20 μ l of single-round competent virus for 1 h at 37°C. Fifty microliters of the serum-peptide-virus mixture was then incubated with Polybrene-treated TZM-bl cells for 72 h at 37°C. The cells were lysed, and cell-associated luciferase activity was determined. The percentage of reduction in neutralization in the presence of peptide was determined at the serum dilution that resulted in 70% inhibition of infection in the absence of peptide, as previously discussed in detail (17).

RESULTS

Characteristics of the envelope immunogens. The clade A envelope immunogens represented sequences with variable length and glycosylation, including viruses with short loop sequences and less glycosylation, viruses with one or the other characteristic, and a virus that was heavily glycosylated and had long loop sequences (Fig. 1). Specifically, Q461e2 had the longest V1-V2 sequence (73 amino acids [aa]) and was the most heavily glycosylated in this region (eight potential N-linked glycosylation sites [PNLGS]), while Q259d2.17 had the shortest (61 aa) and least glycosylated (four PNLGS) V1-V2 region. The lengths and extents of glycosylation of the V4 loop were also different among the four clade A Envs, with Q461e2 having the longest V4 sequence (35 aa) and Q168a2 the shortest (27 aa), while Q769h5 has six PNLGS in V4 and Q259d2.17 has only three PNLGS in V4. In contrast, the lengths and extents of glycosylation of the V3 loop were very similar among the four Envs. Overall, Q259d2.17 was the least glycosylated

(A)

Length of the Variable Regions (amino acids)

	V1V2	V3	V4	V5
SF162	65	35	28	12
Q168a2	61	34	27	11
Q259d2.17	61	35	29	11
Q461e2	73	35	35	10
Q769h5	71	34	33	13

(B)

Number of Potential N-linked Glycosylation Sites

	C1	V1V2	C2	V3	C3	V4	C4	V5	C5	gp41	total
SF162	1	3	6	1	3	5	1	1	0	4	25
Q168a2	2	6	5	1	4	3	1	3	0	5	30
Q259d2.17	1	4	4	1	3	3	1	2	0	4	23
Q461e2	2	8	7	1	4	4	2	1	0	5	34
Q769h5	2	4	6	1	2	6	1	1	0	4	27

(C)

V1V2 region

SF162 CTNLKN-ATNTKSSNWKEMDRGEIKNCSEFKVTTISIRNKMQKEYALFYKLDVVPIDNDN-----TSYKLLINC
 Q168a2 CTNVNN---NTTNVNNNTGWDEERKNCSEFNITTELRLDKRKQVYSLFYKLDVVPIDNSS-----YRLINC
 Q259d2.17 CYNV-----TKSDKITKDMQEEIKNCSEFNITTELRLDKRKQVHSLFYKLDVVPIMGGKN-----DSQYRLINC
 Q769h5 CSNINN-IPNVNASSIPKDVKEEIKNCSEFNITTELRLDKRKQNVYSLFYKLDVVPLETNLKQNSS-HSRYLINC
 Q461e2 CDTWTNNATSTNQTTTPATSEETGVKNCSEFNITTELRLDKRKQVYSLFYKLDVVPQISENNSSNSSNFTQYRLINC

V3 region

SF162 CTRPNNNTRKSIITIGPGRAFAYATGDIIGDIRQAHNC
 Q168a2 CTRPNNNTRKSIIRIGPGQAFYATG-IIGDIRQAYCT
 Q259d2.17 CTRPNNNTRKSVRIIGPGQAFYATDDIIGNIRQAYCT
 Q769h5 CIRPGNNTRKSIHLGPGKVYFATN-IIGDIRQAHNC
 Q461e2 CIRPGNNTRKSVRIIGPGQAFYATGDIIGDIRNAHCV

FIG. 1. Comparison of the SF162 and four clade A envelopes. The amino acid length of the variable Env regions (A) and the number of potential N-linked glycosylation sites per Env region (B) are shown. An alignment of the V1-V2 and V3 regions among the SF162 and the four clade A Envs is also shown (C).

Env (a total of 23 PNLGS) and Q461e2 the most glycosylated Env (a total of 34 PNLGS). For comparison, the clade B SF162 Env has a total of 25 PNLGS, only 3 of which are located in the V1-V2 region. The V1-V2 region of SF162 is 65 aa long and thus is longer than that of Q168a2 and Q259d2.17 but shorter than that of Q461e2 and Q769h5. The length and extent of glycosylation of the SF162 V3 loop are very similar to those of the four clade A Envs.

All of the clade A viral variants from which these four Envs were derived from are rather hard to neutralize (6). Pseudoviruses expressing these Envs were not neutralized (50% neutralization) by heterologous plasmas at a 1:50 plasma dilution, with the exception of Q259d2.17 (the Env with the fewest PNLGS), for which the IC₅₀ with pooled plasma was 66 (6). In addition, only Q168a2 was neutralized by any of the commonly

studied monoclonal antibodies (MAbs) 4E10, 2F5, b12, and 2G12; this variant was weakly neutralized by MAbs 2F5 and 4E10. In contrast, the SF162 virus is highly susceptible to neutralization to heterologous sera and to various MAbs (53, 60). Thus, in addition to the differences in subtype compared to SF162, the clade A variants examined here were also different from SF162 in their overall neutralization susceptibilities.

Development of NAbs. (i) Homologous NAbs. Two weeks following the booster immunization with the recombinant gp140 Envs, the sera were tested for homologous neutralizing activity (Table 1). All the SF162-immunized animals developed potent anti-SF162 NAb titers, as we have previously reported in studies conducted in rabbits immunized with the gene gun immunization methodology and in macaques immunized with

TABLE 1. Titers of homologous serum NABs

Immunogen	Animal	IC ₅₀ NAB titer ^a
SF162	WA493	1,500
	WA494	1,300
	WA495	1,200
	WA496	1,300
	WA497	1,050
	WA498	700
Q259d2.17	WA505	550
	WA506	100
	WA507	—
	WA508	200
	WA509	—
	WA510	—

^a —, neutralization was not recorded at the lowest serum dilution tested (1:20).

the DNA prime following by recombinant Env protein boosting (2, 17). Only three out of six Q259d2.17-immunized animals developed homologous NABs. In contrast, sera from animals immunized with Q168a2, Q461e2, and Q769h5 did not display homologous neutralizing activity at the highest concentration tested (1:20; data not shown).

(ii) Heterologous NABs. None of the SF162-immunized rabbits developed detectable antibodies capable of neutralizing the four clade A viruses (data not shown). In contrast, all animals immunized with Q168a2 or Q461e2, three out of six animals immunized with Q259d2.17, and five out of six animals immunized with Q769h5 developed anti-SF162 NAB responses (Fig. 2A).

Since the clade A Env immunogens appeared to elicit NABs against the clade B isolate SF162, we examined whether other clade B isolates were also susceptible to neutralization by the same immune sera. For these experiments, sera from the animals in each group were pooled and tested against nine clade B isolates: SS1196, QH0692, JRFL, ADA, YU2, 3988, 6101, PAVO, and QH0515.

Sera from the SF162-immunized animals displayed weak neutralizing activity against the heterologous clade B isolate SS1196, but not against any of the other clade B heterologous viruses tested here. Sera from animals immunized with Q168a2 and Q461e2 also neutralized the SS1196 isolate. The remaining clade B viruses tested here were, however, resistant to neutralization by the clade A sera (Fig. 2B and data not shown). As expected from the results shown in Fig. 2A, pooled sera from all five immunized groups neutralized the SF162 virus.

(iii) Easy-to-neutralize clade A isolates. As discussed, with the possible exception of clone Q259d2.17, the clade A Env proteins used here as immunogens were derived from viral clones that were resistant to neutralization by heterologous sera (6). It was, however, previously reported that the patients from whom these viral clones were isolated harbored other viral clones that displayed a more neutralization-susceptible phenotype to heterologous sera (6). We therefore examined whether sera from animals immunized with the four clade A Envs derived from neutralization-resistant viral clones could neutralize the “easy-to-neutralize” viral clones circulating in the same patients (Fig. 3). The sera were tested for neutraliz-

ing activity against pseudoviruses expressing the neutralization-sensitive Env clones: Q168b23, Q259w6, Q769b9, and Q461d1. All four viruses were susceptible to neutralization (to various degrees) by pooled heterologous plasma from 30 individuals, most of whom were infected with subtype A viruses (6), serving here as an internal control. However, only the virus expressing Env Q461d1 was found to be susceptible to neutralization by sera from the Q168a2-, Q461e2-, and Q259d2.17-immunized animals. This virus was also susceptible to neutralization by serum antibodies elicited by the clade B SF162 immunogen. Interestingly, Q461d1 was resistant to neutralization by sera from the Q769h5-immunized animals.

Epitope mapping. The observation that three of the four clade A Env immunogens did not elicit homologous NABs but did elicit cross-reactive NAB responses against two clade B isolates (SF162 and SS1196) suggests that common neutralization epitopes exist among the clade A and B variants tested, but that the relative exposures of these epitopes on the different Envs differ. We therefore decided to map the epitope specificities of the antibodies elicited by the clade A and SF162 Env immunogens. First, we examined whether the antibodies elicited by the five Env immunogens recognized peptides derived from the V1, V2, and V3 loops of SF162 (Table 2). The antibodies elicited by the SF162 immunogen recognized peptides derived from the homologous V1 and V3 loops, but not peptides derived from the homologous V2 loop, as we previously reported (17). Antibodies elicited by the four clade A Env immunogens recognized only the peptide derived from the V3 loop of SF162, but did not recognize peptides derived from either the V1 or V2 loops of SF162. Notably, the V3 of SF162 was recognized despite significant differences in the amino acid compositions of the clade A and clade B SF162 V3 loops (Fig. 1).

As discussed above, the Q259d2.17 Env was the only clade A immunogen that elicited homologous NABs (Table 1). We therefore next examined the epitope specificity of these antibodies using peptides derived from the V1, V2, and V3 loops of Q259d2.17 (Table 2). All six animals immunized with this Env developed antibodies against the V1 loop and the amino-terminal (Nt) region of the V3 loop. In contrast, only three animals (WA505, WA506, and WA508, the same ones that developed homologous NABs, Fig. 2A) developed antibodies to the carboxy terminal (Ct) region of the V3 loop; five animals (WA505, WA506, WA507, WA508, and WA509) developed responses against the Ct region of the V2 loop; and only one animal (WA505) developed antibodies against the Nt region of the V2 loop. Antibodies against the central (crown) region of the V2 loop were undetectable in these animals. Therefore, while both the V1 and V3 loops are immunogenic on the clade A Q259d2.17 and clade B SF162 Envs, the V2 loop is immunogenic only on the former Env.

Relative contributions of serum anti-V1 and anti-V3 antibodies to neutralization. Although the above ELISA experiments suggested that the four clade A Env immunogens elicited cross-reactive binding antibodies to the SF162 V3 loop, they do not provide information as to whether these antibodies are neutralizing. We next examined the relative contribution of the anti-V1 and anti-V3 antibodies elicited by the SF162 Env immunogen and of the anti-V3 antibodies elicited by the four clade A Env immunogens to the observed neutralization of the

FIG. 2. Neutralization of clade B primary isolate by clade A Env-derived sera. (A) The neutralization susceptibility of the clade B virus SF162 by individual sera from animals immunized with the SF162 and the four clade A Env immunogens is presented. (B) The neutralization susceptibility of SF162 and of six heterologous clade B isolates to pooled sera is shown.

Neutralization of 'easy' to neutralize Clade A viral clones

FIG. 3. Neutralization of genetically related "easy-to-neutralize" clade A viral variants. The neutralization susceptibilities of four viral clones derived from the same patients from which the four clade A Env immunogens were derived are shown. These clones are more easily neutralizable by homologous as well as heterologous clade A sera (6).

SF162 virus by using peptide-competition neutralization assays (Table 3).

The anti-SF162 neutralizing activity of clade A sera could be completely inhibited by preincubating the sera with an SF162 V3-derived peptide (Table 3), even though the V3 loops of SF162 and the four clade A Envs differ between 16% and 19% in amino acid sequence (Fig. 1C). In contrast, only 35% of the neutralizing activity of the SF162-elicited sera against the homologous SF162 virus could be inhibited by preincubating these sera with the homologous SF162 V3 peptide (Table 3). As expected, since the clade A Envs did not elicit antibodies to the V1 loop of SF162, preincubation of clade A sera with an SF162 V1-derived peptide had no effect on the neutralizing potency of the sera against the SF162 virus. Similarly, preincubation of sera from the SF162-immunized animals with the SF162 V1 loop-derived peptide had no effect on the anti-SF162 neutralizing activity of the sera.

As discussed above, three animals immunized with the Q259d2.17 Env immunogen (animals WA505, WA506, and WA508) elicited autologous NABs (Table 1). The animals immunized with the remaining three clade A Env immunogens did not generate homologous NABs. Since the anti-V3 antibodies elicited by Q259d2.17 were responsible for the neutralization of the heterologous clade B SF162 virus (Table 3), we examined whether these antibodies were also responsible for the neutralization of the homologous Q259d2.17 virus (Table 3), using two overlapping autologous V3 peptides (one mapping to the Ct region of V3 and the other to the Nt region of V3). However, the preincubation of sera with either one of the Q259d2.17-derived V3 peptides had no effect on the anti-Q259d2.17 neutralizing activity of sera. Interestingly the preadsorption of the Q259d2.17 sera with the V3Nt, but not the V3Ct, Q259-derived peptide completely eliminated the neutralizing activity of these sera against the SF162 virus (data not

TABLE 2. Relative titers of binding anti-V1, -V2, and -V3 serum antibodies

Serum type and immunogen	Relative titer of serum antibodies to peptide ^a :									
	SF162 peptide				Q259d2.17 peptide					
	V1	V2Nt	V2Ct	V3	V1	V2Nt	V2crown ^b	V2Ct	V3Nt	V3Ct
Pooled serum										
SF162	2,500	—	—	3,200						
Q168a2	—	—	—	1,600						
Q259d2.17	—	—	—	3,000						
Q461e2	—	—	—	3,200						
Q769h5	—	—	—	2,000						
Individual sera										
WA505					200	150	—	350	3,200	1,000
WA506					200	—	—	500	3,500	1,000
WA507					300	—	—	100	1,000	—
WA508					200	—	—	800	3,000	800
WA509					300	—	—	1,000	3,200	—
WA510					150	—	—	—	800	—

^a Assays with SF162-derived peptides were performed with pooled sera from the six animals in each immunization group (SF162, Q168a2, Q259d2.17, Q461e2, and Q769h5). Assays with the Q259d2.17-derived peptides were performed with sera from individual sera from the six animals (WA505 to WA510) immunized with the Q259d2.17 Env immunogen. The sequences of these peptides are presented in Materials and Methods. —, peptide reactivity was not recorded at the lowest serum dilution tested (1:100).

^b The “crown” represents the central region of the loop.

shown). Therefore the anti-V3 antibodies, especially the ones that target the Nt of the V3 loop, that are elicited by the Q259d2.17 Env immunogen can bind to their epitopes on the heterologous clade B SF162 virus, but not on the homologous Q259d2.17 virus. The preincubation of sera of the above-mentioned three animals with the homologous V1 peptide also had no effect on the homologous anti-Q259d2.17 neutralizing activity of sera. These results suggest that either the anti-V1 antibodies elicited by Q259d2.17 (Table 2) do not access their epitopes within the virion-associated Q259d2.17 Env molecules, or these epitopes are not neutralization epitopes in the context of the Q259d2.17 virus.

DISCUSSION

The neutralizing potency, breadth, and epitope specificity of serum antibodies elicited during immunization by four clade A Envs was determined. These Envs were selected for several reasons. First, the viruses from which they were derived were obtained directly from infected individuals during acute infection; second, the overall lengths and extents of glycosylation of their variable regions differ; third, the viral clones from which they were derived from were resistant to serum antibody neutralization, as expected from primary HIV-1 isolates; and fourth, they are non-clade B Envs, and very little is known about the immunogenic properties of non-clade B Env immunogens. Limited immunization studies have been conducted with clade C-derived Envs (63), despite the fact that the antigenic and immunogenic properties of clade C and B Envs may differ (24, 44, 51). Clade A Env immunogens, derived from viruses isolated during chronic infection (92RW020 and 92UG037), have been previously included in polyvalent vaccine formulations (55, 56, 62), but their individual immunogenic properties have not been examined.

Only one (Q259d2.17, derived from the Q259 virus) of the four clade A Env immunogens tested here elicited homologous NAbs. This is reminiscent of results obtained with Env immunogens derived from clade B primary isolates resistant to neutralization, such as JRFL or YU2 (3, 4, 27). The epitope specificities of the homologous NAbs elicited by the Q259d2.17 Env immunogen remain unknown, although our peptide competition experiments indicate that these epitopes do not recognize the V1 nor V3 loops. The reason or reasons why the remaining three clade A Envs tested did not elicit homologous NAbs are not yet known. Envs SF162 and Q259d2.17 had the fewest PNLG (25 for SF162 and 23 for Q259d2.17) and were the two immunogens that elicited autologous NAbs. Therefore, it is possible that the ability of an Env protein to elicit autologous NAbs is linked to the length of the V1-V2 loop

TABLE 3. Contribution of anti-V1 and anti-V3 serum antibodies to the neutralizing potential of sera

Serum type and immunogen ^a	% Contribution to neutralizing potential of sera ^b :				
	SF162 peptide		Q259d2.17 peptide		
	V1	V3	V1	V3Nt	V3Ct
Pooled sera					
SF162	0	35			
Q168a2	0	100			
Q259d2.17	0	100			
Q461e2	0	100			
Q769h5	0	100			
Individual sera					
WA505			0	0	0
WA506			0	0	0
WA508			0	0	0

^a Sera from animals immunized with the indicated immunogens were preincubated, or not, with the indicated peptides.

^b The percentage of reduction of the neutralizing activity of sera following preincubation with peptides is shown. The SF162 peptides were derived from SF162 Env. The Q259d2.17 peptides were derived from Q259d2.17 Env.

and/or the extent of its glycosylation. However, neither the V1-V2 region nor the extent of its glycosylation alone appears to define the ability of an Env protein to elicit autologous NAbs. For example, the V1-V2 regions of both Q168a2 and Q259d2.17 are 61 aa long but only Q259d2.17 elicited autologous NAbs. Similarly Q259d2.17 and Q769h5 have the same number of PNLGS in their V1V2 regions, but only the former elicited homologous NAbs. Most likely, the ability of Env immunogens to elicit NAbs will depend not only on the extent of their glycosylation or the length of their variable regions but also on the precise location of the glycosylation sites and the orientation of the variable regions within the trimeric Env.

Despite the fact that three of four clade A Env immunogens failed to elicit homologous NAbs, all four elicited NAbs against the clade B SF162 and SS1196 viruses. This neutralization was mediated exclusively by anti-V3 antibodies, which suggests that these clade A and B V3 loops share structural similarities. This is consistent with previous studies which reported that anti-V3 antibodies isolated from clade A patients can neutralize certain clade B isolates (25, 26, 35). The fact that the anti-V3 antibodies elicited by our four clade A Env immunogens did not access their epitopes on the corresponding clade A virions, but did so on the surface of the heterologous clade B isolates, suggests that these apparently conserved epitopes are occluded on primary clade A viruses but exposed on "easy-to-neutralize" clade B primary isolates. The SF162 and SS1196 viruses are indeed susceptible to neutralization by anti-V3 antibodies (37), presumably because their V3 loops are not as occluded by glycans and other Env regions, as is the case of neutralization-resistant viruses, irrespective of clade.

The fact that the four clade A Envs immunogens elicited anti-V3 antibodies but that these antibodies did not access their targets on the corresponding virion-associated Envs could be attributed to a greater exposure of the V3 loop on the Env immunogens than on the corresponding virion-associated Env. If this is true, that would mean that structural differences exist between the Env immunogens used here and the corresponding virion-associated Env. Differences between the overall epitope exposures on soluble Env immunogens and virion-associated Envs have been discussed previously (47, 61).

The V3 loops of SF162 and the four clade A Envs tested here differ between 16% and 19% in amino acid sequence. However, certain domains of the V3 loop are identical among the five Envs (Fig. 1C): for example, the NNTR motif at the Nt of the loop, the GPG motif at the "crown" of the loop, and the FYAT motif immediately at the Nt side of the "crown." It is therefore possible that the anti-V3 cross-NAbs elicited by these Envs target one or more of these motifs. Alternatively, it is possible that the anti-V3 antibodies elicited by the clade A Envs recognize conserved conformational epitopes among clade A and B viruses. In fact Gorny et al. reported that antibodies to conformational V3 epitopes can neutralize viruses from different clades (25). At the moment, we do not know whether neutralization of the "easy-to-neutralize" Q461d1 variant by the serum antibodies elicited by the clade B SF162 and three of four clade A Envs (Q168a2, Q259d2.17 and Q461e2) is mediated by anti-V3 antibodies or antibodies that bind to other regions of Env, since the overall conformation of the Q461d1 Env is more "open" and the virus is globally susceptible to neutralization (7).

Previously it was reported that immunization of macaques with SF162 Env gp140 results in the generation of binding anti-V1 and anti-V3 antibodies, in most animals, and that both types of antibodies contribute to the homologous neutralizing activity of sera (15, 17). In this study, although the anti-V3 antibodies elicited during immunization with the SF162 Env immunogen displayed the expected anti-SF162 neutralizing activity, the anti-V1 antibodies did not appear to contribute to the overall anti-SF162 neutralizing activity of sera. Several reasons could account for this. For example, different forms of Env immunogens were used. Previously, the gp140 form of Env was used during both the priming and the boosting phases of immunization, while here the gp160 form was used during the priming phase. An additional variable was that in the previous studies we used DNA during the priming phase of immunization, while here we used vaccinia virus. Also, purified soluble gp140 trimers were used during the boosting phase of immunization previously, while here a mixture of monomers, trimers and higher-molecular-weight aggregates were used during boosting. Finally, previously we used MF59C as an adjuvant, while here we used Freund's as the adjuvant. Defining which of these possibilities is responsible for the differential neutralizing activity of the anti-V1 loop antibodies in this and the previous study is something we are planning to investigate in the near future.

The elicitation of cross-reactive NAbs against HIV by immunization will depend on several factors, including the immunogen used, adjuvant, and immunization protocol. The "prime boost" immunization protocol is very effective in the elicitation of anti-Env antibodies. However, there are limitations to this protocol which potentially could in part be responsible for the lack of generation of autologous NAbs by the clade A Envs used here. Also, the use of Freund's as an adjuvant during our "booster" immunization may be another limitation. Other adjuvants appear to be more effective in improving the immunogenicity of Env (38, 71).

Overall, our study indicates that the immunogenicity of conserved Env regions on clade A Env immunogens will be as weak as that on clade B-derived Env immunogens. Our findings also suggest that the immunogenicity of Envs from "early" transmitted viruses will not be different from Envs derived from viruses isolated during chronic infection. However, since we only used one virus from chronic infection, SF162, further studies need to be performed to verify this point. Cross-reactive anti-V3 antibodies will be elicited by clade A Env immunogens, but they will display limited cross-neutralizing reactivities due to the occluded nature of the virion-associated V3 loop on clade A viruses. Even homologous NAbs will be hard to elicit with Envs derived from primary neutralization-resistant clade A viruses, as observed with clade B-derived Envs. Therefore, similar problems face the development of immunogens capable of eliciting cross-reactive NAbs against clade B and A viruses.

ACKNOWLEDGMENTS

We thank colleagues at Covance Research Products, Inc. (Denver, PA), for conducting the rabbit immunization studies. We thank Catherine A. Blish, George Sellhorn, Aaron Wallace, and Jakob Armann for critical reading of the manuscript.

These studies were supported by HIVRAD grant P01 AI054564 (S.L.-H.). We also acknowledge financial support by the M. J. Murdock Charitable Trust and the J. B. Pendleton Charitable Trust.

REFERENCES

- Back, N. K. T., L. Smit, J.-J. De Jong, W. Keulen, M. Schutten, J. Goudsmit, and M. Tersmette. 1994. An N-glycan within the human immunodeficiency virus type 1 gp120 V3 loop affects virus neutralization. *Virology* **199**:431–438.
- Barnett, S. W., S. Lu, I. Srivastava, S. Cherpelis, A. Gettiffe, J. Blanchard, S. Wang, I. Mboudjeka, L. Leung, Y. Lian, A. Fong, C. Buckner, A. Ly, S. Hilt, J. Ulmer, C. T. Wild, J. R. Mascola, and L. Stamatatos. 2001. The ability of an oligomeric human immunodeficiency virus type 1 (HIV-1) envelope antigen to elicit neutralizing antibodies against primary HIV-1 isolates is improved following partial deletion of the second hypervariable region. *J. Virol.* **75**:5526–5540.
- Beddows, S., M. Franti, A. K. Dey, M. Kirschner, S. P. Iyer, D. C. Fisch, T. Ketas, E. Yuste, R. C. Desrosiers, P. J. Klasse, P. J. Maddon, W. C. Olson, and J. P. Moore. 2007. A comparative immunogenicity study in rabbits of disulfide-stabilized, proteolytically cleaved, soluble trimeric human immunodeficiency virus type 1 gp140, trimeric cleavage-defective gp140 and monomeric gp120. *Virology* **360**:329–340.
- Beddows, S., N. Schülke, M. Kirschner, K. Barnes, M. Franti, E. Michael, T. Ketas, R. W. Sanders, P. J. Maddon, W. C. Olson, and J. P. Moore. 2005. Evaluating the immunogenicity of a disulfide-stabilized, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. *J. Virol.* **79**:8812–8827.
- Binley, J. M., R. W. Sanders, B. Clas, N. Schuelke, A. Master, Y. Guo, F. Kajumo, D. J. Anselma, P. J. Maddon, W. C. Olson, and J. P. Moore. 2000. A recombinant human immunodeficiency virus type 1 envelope glycoprotein complex stabilized by an intermolecular disulfide bond between the gp120 and gp41 subunits is an antigenic mimic of the trimeric virion-associated structure. *J. Virol.* **74**:627–643.
- Blish, C. A., R. Nedellec, K. Mandalia, D. E. Mosier, and J. Overbaugh. 2007. HIV-1 subtype A envelope variants from early in infection have variable sensitivity to neutralization and to inhibitors of viral entry. *AIDS* **21**: 693–702.
- Blish, C. A., M. A. Nguyen, and J. Overbaugh. 2008. Enhancing exposure of HIV-1 neutralization epitopes through mutations in gp41. *PLoS Med.* **5**:e9.
- Bolmstedt, A., S. Sjolander, J. E. Hansen, L. Akerblom, A. Hemming, S. L. Hu, B. Morein, and S. Olofsson. 1996. Influence of N-linked glycans in V4-V5 region of human immunodeficiency virus type 1 glycoprotein gp160 on induction of a virus-neutralizing humoral response. *J. Acquir. Immune Defic. Syndr. Hum. Retrovir.* **12**:213–220.
- Buonaguro, L., M. L. Tornesello, and F. M. Buonaguro. 2007. Human immunodeficiency virus type 1 subtype distribution in the worldwide epidemic: pathogenetic and therapeutic implications. *J. Virol.* **81**:10209–10219.
- Bures, R., A. Gaitan, T. Zhu, C. Graziosi, K. M. McGrath, J. Tartaglia, P. Caudrelier, R. El Habib, M. Klein, A. Lazzarin, D. M. Stablein, M. Deers, L. Corey, M. L. Greenberg, D. H. Schwartz, and D. C. Montefiori. 2000. Immunization with recombinant canarypox vectors expressing membrane-anchored glycoprotein 120 followed by glycoprotein 160 boosting fails to generate antibodies that neutralize R5 primary isolates of human immunodeficiency virus type 1. *AIDS Res. Hum. Retrovir.* **16**:2019–2035.
- Burke, B., N. R. Derby, Z. Kraft, C. J. Saunders, C. Dai, N. Llewellyn, I. Zharkikh, L. Vojtech, T. Zhu, I. K. Srivastava, S. W. Barnett, and L. Stamatatos. 2006. Viral evolution in macaques coinfecting with CCR5- and CXCR4-tropic SHIVs in the presence or absence of vaccine-elicited anti-CCR5 SHIV neutralizing antibodies. *Virology* **355**:138–151.
- Cao, J., N. Sullivan, E. Desjardins, C. Parolin, J. Robinson, R. Wyatt, and J. Sodroski. 1997. Replication and neutralization of human immunodeficiency virus type 1 lacking the V1 and V2 variable loops of the gp120 envelope glycoprotein. *J. Virol.* **71**:9808–9812.
- Center, R. J., J. Lebowitz, R. D. Leapman, and B. Moss. 2004. Promoting trimerization of soluble human immunodeficiency virus type 1 (HIV-1) Env through the use of HIV-1/simian immunodeficiency virus chimeras. *J. Virol.* **78**:2265–2276.
- Chakrabarti, S., J. R. Sisler, and B. Moss. 1997. Compact, synthetic, vaccinia virus early/late promoter for protein expression. *BioTechniques* **23**:1094–1097.
- Ching, L. K., G. Vlachogiannis, K. A. Bosch, and L. Stamatatos. 2008. The first hypervariable region of the gp120 Env glycoprotein defines the neutralizing susceptibility of heterologous human immunodeficiency virus type 1 isolates to neutralizing antibodies elicited by the SF162gp140 immunogen. *J. Virol.* **82**:949–956.
- Chohan, B., D. Lang, M. Sagar, B. Korber, L. Lavreys, B. Richardson, and J. Overbaugh. 2005. Selection for human immunodeficiency virus type 1 envelope glycosylation variants with shorter V1-V2 loop sequences occurs during transmission of certain genetic subtypes and may impact viral RNA levels. *J. Virol.* **79**:6528–6531.
- Derby, N. R., Z. Kraft, E. Kan, E. T. Crooks, S. W. Barnett, I. K. Srivastava, J. M. Binley, and L. Stamatatos. 2006. Antibody responses elicited in macaques immunized with human immunodeficiency virus type 1 (HIV-1) SF162-derived gp140 envelope immunogens: comparison with those elicited during homologous simian/human immunodeficiency virus SHIV_{SF162P4} and heterologous HIV-1 infection. *J. Virol.* **80**:8745–8762.
- Derdeyn, C. A., J. M. Decker, F. Bibollet-Ruche, J. L. Mokili, M. Muldoon, S. A. Denham, M. L. Heil, F. Kasolo, R. Musonda, B. H. Hahn, G. M. Shaw, B. T. Korber, S. Allen, and E. Hunter. 2004. Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission. *Science* **303**:2019–2022.
- Dey, B., M. Pancera, K. Svehla, Y. Shu, S.-H. Xiang, J. Vainshtein, Y. Li, J. Sodroski, P. D. Kwong, J. R. Mascola, and R. Wyatt. 2007. Characterization of human immunodeficiency virus type 1 monomeric and trimeric gp120 glycoproteins stabilized in the CD4-bound state: antigenicity, biophysics, and immunogenicity. *J. Virol.* **81**:5579–5593.
- Doria-Rose, N. A., G. H. Learn, A. G. Rodrigo, D. C. Nickle, F. Li, M. Mahalanabis, M. T. Hensel, S. McLaughlin, P. F. Edmonson, D. Montefiori, S. W. Barnett, N. L. Haigwood, and J. I. Mullins. 2005. Human immunodeficiency virus type 1 subtype B ancestral envelope protein is functional and elicits neutralizing antibodies in rabbits similar to those elicited by a circulating subtype B envelope. *J. Virol.* **79**:11214–11224.
- Earl, P. L., W. Sugiura, D. C. Montefiori, C. C. Broder, S. A. Lee, C. Wild, J. Lifson, and B. Moss. 2001. Immunogenicity and protective efficacy of oligomeric human immunodeficiency virus type 1 gp140. *J. Virol.* **75**:645–653.
- Farzan, M., H. Choe, E. Desjardins, Y. Sun, J. Kuhn, J. Cao, D. Archambault, P. Kolchinsky, M. Koch, R. Wyatt, and J. Sodroski. 1998. Stabilization of human immunodeficiency virus type 1 envelope glycoprotein trimers by disulfide bonds introduced into the gp41 glycoprotein ectodomain. *J. Virol.* **72**:7620–7625.
- Gao, F., E. A. Weaver, Z. Lu, Y. Li, H.-X. Liao, B. Ma, S. M. Alam, R. M. Scearce, L. L. Sutherland, J.-S. Yu, J. M. Decker, G. M. Shaw, D. C. Montefiori, B. T. Korber, B. H. Hahn, and B. F. Haynes. 2005. Antigenicity and immunogenicity of a synthetic human immunodeficiency virus type 1 group M consensus envelope glycoprotein. *J. Virol.* **79**:1154–1163.
- Gnanakaran, S., D. Lang, M. Daniels, T. Bhattacharya, C. A. Derdeyn, and B. Korber. 2007. Clade-specific differences between human immunodeficiency virus type 1 clades B and C: diversity and correlations in C3-V4 regions of gp120. *J. Virol.* **81**:4886–4891.
- Gorny, M. K., C. Williams, B. Volsky, K. Revesz, S. Cohen, V. R. Polonis, W. J. Honnen, S. C. Kayman, C. Krachmarov, A. Pinter, and S. Zolla-Pazner. 2002. Human monoclonal antibodies specific for conformation-sensitive epitopes of V3 neutralize human immunodeficiency virus type 1 primary isolates from various clades. *J. Virol.* **76**:9035–9045.
- Gorny, M. K., C. Williams, B. Volsky, K. Revesz, X.-H. Wang, S. Burda, T. Kimura, F. A. J. Konings, A. Nadas, C. A. Anyangwe, P. Nyambi, C. Krachmarov, A. Pinter, and S. Zolla-Pazner. 2006. Cross-clade neutralizing activity of human anti-V3 monoclonal antibodies derived from the cells of individuals infected with non-B clades of human immunodeficiency virus type 1. *J. Virol.* **80**:6865–6872.
- Grundner, C., Y. Li, M. Louder, J. Mascola, X. Yang, J. Sodroski, and R. Wyatt. 2005. Analysis of the neutralizing antibody response elicited in rabbits by repeated inoculation with trimeric HIV-1 envelope glycoproteins. *Virology* **331**:33–46.
- Haigwood, N. L., P. L. Nara, E. Brooks, G. A. V. Nest, G. Ott, K. W. Higgins, N. Dunlop, C. J. Scandella, J. W. Eichberg, and K. S. Steimer. 1992. Native but not denatured recombinant human immunodeficiency virus type 1 gp120 generates broad-spectrum neutralizing antibodies in baboons. *J. Virol.* **66**: 172–182.
- Hanson, C. V. 1994. Measuring vaccine-induced HIV neutralization: report of a workshop. *AIDS Res. Hum. Retrovir.* **10**:645–648.
- Hu, S. L., S. G. Kosowski, and J. M. Dalrymple. 1986. Expression of AIDS virus envelope gene in recombinant vaccinia viruses. *Nature* **320**:537–540.
- Johnson, W. E., J. Morgan, J. Reitter, B. A. Puffer, S. Czajak, R. W. Doms, and R. C. Desrosiers. 2002. A replication-competent, neutralization-sensitive variant of simian immunodeficiency virus lacking 100 amino acids of envelope. *J. Virol.* **76**:2075–2086.
- Johnson, W. E., H. Sanford, L. Schwall, D. R. Burton, P. W. H. I. Parren, J. E. Robinson, and R. C. Desrosiers. 2003. Assorted mutations in the envelope gene of simian immunodeficiency virus lead to loss of neutralization resistance against antibodies representing a broad spectrum of specificities. *J. Virol.* **77**:9993–10003.
- Klaniécki, J., T. Dykers, B. Travis, R. Schmitt, M. Wain, A. Watson, P. Sridhar, J. McClure, B. Morein, J. T. Ulrich, S.-L. Hu, and J. Lewis. 1991. Cross-neutralizing antibodies in rabbits immunized with HIV-1 gp160 purified from simian cells infected with a recombinant vaccinia virus. *AIDS Res. Hum. Retrovir.* **7**:791–798.
- Koch, M., M. Pancera, P. D. Kwong, P. Kolchinsky, C. Grundner, L. Wang, W. A. Hendrickson, J. Sodroski, and R. Wyatt. 2003. Structure-based, targeted deglycosylation of HIV-1 gp120 and effects on neutralization sensitivity and antibody recognition. *Virology* **313**:387–400.
- Krachmarov, C., A. Pinter, W. J. Honnen, M. K. Gorny, P. N. Nyambi, S. Zolla-Pazner, and S. C. Kayman. 2005. Antibodies that are cross-reactive for human immunodeficiency virus type 1 clade A and clade B V3 domains are common in patient sera from Cameroon, but their neutralization activity is usually restricted by epitope masking. *J. Virol.* **79**:780–790.
- Kraft, Z., N. R. Derby, R. A. McCaffrey, R. Niec, W. M. Blay, N. L. Haigwood,

- E. Moysi, C. J. Saunders, T. Wrin, C. J. Petropoulos, M. J. McElrath, and L. Stamatatos. 2007. Macaques infected with a CCR5-tropic simian/human immunodeficiency virus (SHIV) develop broadly reactive anti-HIV neutralizing antibodies. *J. Virol.* **81**:6402–6411.
37. Li, M., F. Gao, J. R. Mascola, L. Stamatatos, V. R. Polonis, M. Koutsoukos, G. Voss, P. Goepfert, P. Gilbert, K. M. Greene, M. Bilska, D. L. Kothe, J. F. Salazar-Gonzalez, X. Wei, J. M. Decker, B. H. Hahn, and D. C. Montefiori. 2005. Human immunodeficiency virus type 1 *env* clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. *J. Virol.* **79**:10108–10125.
38. Li, Y., K. Svehla, N. L. Mathy, G. Voss, J. R. Mascola, and R. Wyatt. 2006. Characterization of antibody responses elicited by human immunodeficiency virus type 1 primary isolate trimeric and monomeric envelope glycoproteins in selected adjuvants. *J. Virol.* **80**:1414–1426.
39. Long, E. M., S. M. Rainwater, L. Lavreys, K. Mandaliya, and J. Overbaugh. 2002. HIV type 1 variants transmitted to women in Kenya require the CCR5 coreceptor for entry, regardless of the genetic complexity of the infecting virus. *AIDS Res. Hum. Retrovir.* **18**:567–576.
40. Ly, A., and L. Stamatatos. 2000. V2 loop glycosylation of the human immunodeficiency virus type 1 SF162 envelope facilitates interaction of this protein with CD4 and CCR5 receptors and protects the virus from neutralization by anti-V3 loop and anti-CD4 binding site antibodies. *J. Virol.* **74**:6769–6776.
41. Malenbaum, S. E., D. Yang, L. Cavacini, M. Posner, J. Robinson, and C. Cheng-Mayer. 2000. The N-terminal V3 loop glycan modulates the interaction of clade A and B human immunodeficiency virus type 1 envelopes with CD4 and chemokine receptors. *J. Virol.* **74**:11008–11016.
42. Mascola, J. R., S. W. Snyder, O. S. Weislow, S. M. Belay, R. B. Belshe, D. H. Schwartz, M. L. Clements, R. Dolin, B. S. Graham, G. J. Gorse, M. C. Keefer, M. J. McElrath, M. C. Walker, K. F. Wagner, J. G. McNeil, F. E. McCutchan, D. S. Burke, and the NIAID AIDS Vaccine Evaluation Group. 1996. Immunization with envelope subunit vaccine products elicits neutralizing antibodies against laboratory-adapted but not primary isolates of human immunodeficiency virus type 1. *J. Infect. Dis.* **173**:340–348.
43. McCaffrey, R. A., C. Saunders, M. Hensel, and L. Stamatatos. 2004. N-linked glycosylation of the V3 loop and the immunologically silent face of gp120 protects human immunodeficiency virus type 1 SF162 from neutralization by anti-gp120 and anti-gp41 antibodies. *J. Virol.* **78**:3279–3295.
44. Moore, P. L., E. S. Gray, I. A. Choge, N. R. R. Chhabe, K. Mlisana, S. S. Abdool Karim, C. Williamson, L. Morris, and the CAPRISA 002 Study Team. 2008. The C3-V4 region is a major target of autologous neutralizing antibodies in human immunodeficiency virus type 1 subtype C infection. *J. Virol.* **82**:1860–1869.
45. Nabatov, A. A., G. Pollakis, T. Linnemann, A. Kliphuis, M. I. Chalaby, and W. A. Paxton. 2004. Intrapatent alterations in the human immunodeficiency virus type 1 gp120 V1V2 and V3 regions differentially modulate coreceptor usage, virus inhibition by CC/CX chemokines, soluble CD4, and the b12 and 2G12 monoclonal antibodies. *J. Virol.* **78**:524–530.
46. Overbaugh, J., and L. M. Rudensey. 1992. Alterations in potential sites for glycosylation predominate during evolution of the simian immunodeficiency virus envelope gene in macaques. *J. Virol.* **66**:5937–5948.
47. Pancera, M., J. Lebowitz, A. Schön, P. Zhu, E. Freire, P. D. Kwong, K. H. Roux, J. Sodroski, and R. Wyatt. 2005. Soluble mimetics of human immunodeficiency virus type 1 viral spikes produced by replacement of the native trimerization domain with a heterologous trimerization motif: characterization and ligand binding analysis. *J. Virol.* **79**:9954–9969.
48. Pinter, A., W. J. Honnen, Y. He, M. K. Gorny, S. Zolla-Pazner, and S. C. Kayman. 2004. The V1/V2 domain of gp120 is a global regulator of the sensitivity of primary human immunodeficiency virus type 1 isolates to neutralization by antibodies commonly induced upon infection. *J. Virol.* **78**:5205–5215.
49. Reitter, J. N., R. E. Means, and R. C. Desrosiers. 1998. A role for carbohydrates in immune evasion in AIDS. *Nat. Med.* **4**:679–684.
50. Richardson, T. M., Jr., B. L. Strykowski, C. C. Broder, J. A. Hoxie, J. R. Mascola, P. L. Earl, and R. W. Doms. 1996. Humoral response to oligomeric human immunodeficiency virus type 1 envelope protein. *J. Virol.* **70**:753–762.
51. Rong, R., S. Gnanakaran, J. M. Decker, F. Bibollet-Ruche, J. Taylor, J. N. Sfakianos, J. L. Mokili, M. Muldoon, J. Mulenga, S. Allen, B. H. Hahn, G. M. Shaw, J. L. Blackwell, B. T. Korber, E. Hunter, and C. A. Derdeyn. 2007. Unique mutational patterns in the envelope $\alpha 2$ amphipathic helix and acquisition of length in gp120 hypervariable domains are associated with resistance to autologous neutralization of subtype C human immunodeficiency virus type 1. *J. Virol.* **81**:5658–5668.
52. Sanders, R. W., M. Vesanen, N. Schuelke, A. Master, L. Schiffrer, R. Kalyanaraman, M. Paluch, B. Berkhout, P. J. Maddon, W. C. Olson, M. Lu, and J. A. Moore. 2002. Stabilization of the soluble, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. *J. Virol.* **76**:8875–8889.
53. Saunders, C. J., R. A. McCaffrey, I. Zharkikh, Z. Kraft, S. E. Malenbaum, B. Burke, C. Cheng-Mayer, and L. Stamatatos. 2005. The V1, V2, and V3 regions of the human immunodeficiency virus type 1 envelope differentially affect the viral phenotype in an isolate-dependent manner. *J. Virol.* **79**:9069–9080.
54. Schülke, N., M. S. Vesanen, R. W. Sanders, P. Zhu, M. Lu, D. J. Anselma, A. R. Villa, P. W. I. Parren, J. M. Binley, K. H. Roux, P. J. Maddon, J. P. Moore, and W. C. Olson. 2002. Oligomeric and conformational properties of a proteolytically mature, disulfide-stabilized human immunodeficiency virus type 1 gp140 envelope glycoprotein. *J. Virol.* **76**:7760–7776.
55. Seaman, M. S., D. F. Leblanc, L. E. Grandpre, M. T. Bartman, D. C. Montefiori, N. L. Letvin, and J. R. Mascola. 2007. Standardized assessment of NAb responses elicited in rhesus monkeys immunized with single- or multi-clade HIV-1 envelope immunogens. *Virology* **367**:175–186.
56. Seaman, M. S., S. Santra, M. H. Newberg, V. Philippon, K. Manson, L. Xu, R. S. Gelman, D. Panicali, J. R. Mascola, G. J. Nabel, and N. L. Letvin. 2005. Vaccine-elicited memory cytotoxic T lymphocytes contribute to *Mamu-A*01*-associated control of simian/human immunodeficiency virus 89.6P replication in rhesus monkeys. *J. Virol.* **79**:4580–4588.
57. Shioda, T., J. A. Levy, and C. Cheng-Mayer. 1991. Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. *Nature (London)* **349**:167–169.
58. Srivastava, I. K., L. Stamatatos, E. Kan, M. Vajdy, Y. Lian, S. Hilt, L. Martin, C. Vita, P. Zhu, K. H. Roux, L. Vojtech, D. C. Montefiori, J. Donnelly, J. B. Ulmer, and S. W. Barnett. 2003. Purification, characterization, and immunogenicity of a soluble trimeric envelope protein containing a partial deletion of the V2 loop derived from SF162, an R5-tropic human immunodeficiency virus type 1 isolate. *J. Virol.* **77**:11244–11259.
59. Srivastava, I. K., L. Stamatatos, H. Legg, E. Kan, A. Fong, S. R. Coates, L. Leung, M. Wininger, J. J. Donnelly, J. B. Ulmer, and S. W. Barnett. 2002. Purification and characterization of oligomeric envelope glycoprotein from a primary R5 subtype B human immunodeficiency virus. *J. Virol.* **76**:2835–2847.
60. Stamatatos, L., and C. Cheng-Mayer. 1998. An envelope modification that renders a primary, neutralization-resistant, clade B human immunodeficiency virus type 1 isolate highly susceptible to neutralization by sera from other clades. *J. Virol.* **72**:7840–7845.
61. Stamatatos, L., M. Lim, and C. Cheng-Mayer. 2000. Generation and structural analysis of soluble oligomeric envelope proteins derived from neutralization-resistant and neutralization-susceptible primary HIV-1 isolates. *AIDS Res. Hum. Retrovir.* **16**:981–994.
62. Wang, S., R. Pal, J. R. Mascola, T. H. Chou, I. Mboudjeka, S. Shen, Q. Liu, S. Whitney, T. Keen, B. C. Nair, V. S. Kalyanaraman, P. Markham, and S. Lu. 2006. Polyvalent HIV-1 Env vaccine formulations delivered by the DNA priming plus protein boosting approach are effective in generating neutralizing antibodies against primary human immunodeficiency virus type 1 isolates from subtypes A, B, C, D and E. *Virology* **350**:34–47.
63. Wu, L., Z. Y. Yang, L. Xu, B. Welcher, S. Winfrey, Y. Shao, J. R. Mascola, and G. J. Nabel. 2006. Cross-clade recognition and neutralization by the V3 region from clade C human immunodeficiency virus-1 envelope. *Vaccine* **24**:4995–5002.
64. Xu, R., I. K. Srivastava, C. E. Greer, I. Zarkikh, Z. Kraft, L. Kuller, J. M. Polo, S. W. Barnett, and L. Stamatatos. 2006. Characterization of immune responses elicited in macaques immunized sequentially with chimeric VEE/SIN alphavirus replicon particles expressing SIVGag and/or HIVEnv and with recombinant HIVgp140Env protein. *AIDS Res. Hum. Retrovir.* **22**:1022–1030.
65. Xu, R., I. K. Srivastava, L. Kuller, I. Zarkikh, Z. Kraft, Z. Fagrouch, N. L. Letvin, J. L. Heeny, S. W. Barnett, and L. Stamatatos. 2006. Immunization with HIV-1 SF162-derived envelope gp140 proteins does not protect macaques from heterologous simian-human immunodeficiency virus SHIV89.6P infection. *Virology* **349**:276–289.
66. Yang, X., L. Florin, M. Farzan, P. Kolchinsky, P. D. Kwong, J. Sodroski, and R. Wyatt. 2000. Modifications that stabilize human immunodeficiency virus envelope glycoprotein trimers in solution. *J. Virol.* **74**:4746–4754.
67. Yang, X., J. Lee, E. M. Mahony, P. D. Kwong, R. Wyatt, and J. Sodroski. 2002. Highly stable trimers formed by human immunodeficiency virus type 1 envelope glycoproteins fused with the trimeric motif of T4 bacteriophage fibritin. *J. Virol.* **76**:4634–4642.
68. Yang, X., R. Wyatt, and J. Sodroski. 2001. Improved elicitation of neutralizing antibodies against primary human immunodeficiency viruses by soluble stabilized envelope glycoprotein trimers. *J. Virol.* **75**:1165–1171.
69. Ye, Y., Z. H. Si, J. P. Moore, and J. Sodroski. 2000. Association of structural changes in the V2 and V3 loops of the gp120 envelope glycoprotein with acquisition of neutralization resistance in a simian-human immunodeficiency virus passaged in vivo. *J. Virol.* **74**:11955–11962.
70. Zarling, J. M., W. Morton, P. A. Moran, J. McClure, S. G. Kosowski, and S. L. Hu. 1986. T-cell responses to human AIDS virus in macaques immunized with recombinant vaccinia viruses. *Nature* **323**:344–346.
71. Zhang, P. F., F. Cham, M. Dong, A. Choudhary, P. Bouma, Z. Zhang, Y. Shao, Y. R. Feng, L. Wang, N. Mathy, G. Voss, C. C. Broder, and G. V. Quinnan, Jr. 2007. Extensively cross-reactive anti-HIV-1 neutralizing antibodies induced by gp140 immunization. *Proc. Natl. Acad. Sci. USA* **104**:10193–10198.